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TITLE: NF- κ B-Mediated Repression of GADD153/CHOP: A Role in
Breast Cancer Initiation

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13. ABSTRACT (Maximum 200 Words) Cells exposed to stresses such as nutrient deprivation, hypoxia, ultraviolet light and environmental toxicants that damage DNA or disrupt endoplasmic reticulum function show induced expression of GADD153/CHOP, a pro-apoptotic C/EBP family transcription factor. Interestingly, cellular stress also induces the activity of the transcription factor NF- κ B, which promotes cell survival by inducing transcription of anti-apoptotic proteins, such as Bcl-2, Bcl-XL, x-IAP, and c-IAP-2. GADD153 mRNA expression was previously shown in this laboratory to be inhibited by the p65 transactivating subunit of NF- κ B. Therefore, cell expressing constitutively active NF- κ B, as is the case in 30% of breast cancers, may be predisposed to transformation following cellular stress due to p65-mediated inhibition of GADD153 expression. To address this possibility, we have generated immortalized mammary epithelial cell line MCF-10A overexpressing p65. p65 overexpressing cells showed hallmarks of epithelial to mesenchymal transition (EMT) including reduced expression of E-cadherin and Occludin and increased expression of Vimentin. Although the role of EMT in breast cancer initiation is not known, it is essential for invasive growth and motility of breast cancer cells. We also observed that the environmental toxicant methyl methanesulfonate (MMS) induces the expression of the transcription repressor Snail, which has been implicated in EMT. Furthermore, in transient transfection assay, we observed Snail-mediated repression of GADD153 expression. Interestingly, Sequences within the first exon of GADD153 involve its repression by both NF- κ B and Snail. Because Snail expression in Drosophila is regulated by NF- κ B homologue dorsal, we propose that NF- κ B similarly regulates expression of Snail family transcription repressors, which in turn promotes breast cancer progression through enhanced EMT.				
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INTRODUCTION:

Understanding the mechanism of breast cancer initiation is critical for developing any chemoprevention strategies. Normal mammary epithelial cells from which cancer usually originate have limited life span. Immortalization is the first step that leads to continuous growth of mammary epithelial cells (1). Cell cycle regulatory, anti-apoptotic and pro-apoptotic proteins play a significant role in immortalization process (1). Immortalized cells attain cancerous growth properties (transformation) due to additional mutations that lead to either loss of tumor suppressor genes or activation of oncogenes. The transcription factor NF- κ B promotes both immortalization and transformation by controlling the expression of cell cycle regulatory, anti-apoptotic and pro-apoptotic genes (2). NF- κ B is usually sequestered in the cytoplasm of resting cells through its association with inhibitor of kappaB proteins and translocates to nucleus upon exposure of cells to cytokines and growth factors (3). NF- κ B then binds to response elements and induces the expression of genes involved in invasion, metastasis and chemotherapy resistance. We and others have shown that NF- κ B is constitutively active in breast cancer and is responsible for overexpression of several anti-apoptotic genes as well as repression of the pro-apoptotic gene GADD153/CHOP (4-7). GADD153 is induced when DNA is damaged or cells are under stress. Depending on the extent of damage, cells either repair DNA damage and survive or die. GADD153 is believed to promote death of cells with severe damage, thereby limiting accumulation of cells with mutations (8). Thus, GADD153 is likely to play a role in preventing breast cancer initiation. Because NF- κ B reduces GADD153 expression, it is possible that cells that contain constitutively active NF- κ B will survive after DNA damage. Such cells with damaged DNA are more prone for transformation. This award is to test this possibility. There are three aims in this proposal: 1) To determine whether inhibition of GADD153 by NF- κ B is essential for survival and/or transformation of MCF-10A cells when exposed to MMS or grown under nutrient-deprived condition. 2) To determine whether inhibition of GADD153 by NF- κ B leads to altered activity of the transcription factor C/EBP β and differentiation of MCF-10A cells. 3) To determine the influence of p53 on the anti-apoptotic function of NF- κ B in MCF-10A cells grown under nutrient-deprived condition or exposed to MMS.

BODY:

Specific Aim I:

Task 1:(months 1-5) Establish MCF10A cells overexpressing p65NLS50 or ras and characterize them with respect to constitutive NF- κ B activity.

Results: MCF10A cells were transduced with the bicistronic retroviral vector pQCXIP (puromycin resistance) containing the p65 coding sequence (with a modification of nuclear translocation signal to that of p50). Western blot analysis confirmed overexpression of p65 in cells transduced with p65 coding sequence containing virus (MCF10/p65NLS50) compared to MCF10/pQ vector control cells. Enhanced NF- κ B DNA binding activity was observed in MCF10/p65NLS50 compared to MCF10/pQ cells (Fig. 1). We have also obtained MCF-10A cells overexpressing activated Ras.

Initial Northern blot analysis showed that exposure to MMS for 4 hrs induces GADD153 expression in MCF10/pQ vector control cells; however a lower level of expression was observed in MCF10A/p65 cells. In a concurrent study with MDA-MB-

231 cells overexpressing I κ B α SR, we observed NF- κ B-dependent repression of calcium ionopore- and MMS-induced GADD153 expression in these cancer cells (Fig. 2). Thus, there is a cell type specific difference in inducible GADD153 expression and its repression by NF- κ B. We will focus our future studies on the effect of NF- κ B on induction of GADD153 upon nutrient deprivation.

Task 2: (Months 6-8) Determine MMS-inducible and nutrient-deprivation inducible expression of GADD153 in MCF10/p65NLS50 cells by Northern and Western blots. Determine apoptosis by Annexin V labeling, PARP cleavage and DNA laddering.

Results: Initial cell viability experiments were carried out using the MTT assay and clonogenic assays. MCF10A/p65NLS50 cells treated with MMS (0 to 10 mM) for 3 to 24 hrs did not show a significant difference in cell viability, as measured by MTT assay compared to vector control cells.

Because p65 overexpression does not appear to alter cell survival in response to MMS treatment, we will focus our efforts on nutrient deprivation. We have also found that NF- κ B overexpression has no effect on GADD153 expression in cells exposed to other environmental toxicants such as benzo-a-pyrene and 6-nitrochrysene.

Task 3: Months (9-15) Determine the susceptibility of MCF-10A, ras and p65NLS50 cells to MMS-induced transformation. This will be achieved by growing cells in soft agar and matrigel.

Results: Matrigel assays have been initiated and we are currently standardizing procedures to obtain sections for immunohistochemical staining. We will use foci formation under low serum (0.1%) without EGF as an assay for transformation as described recently (9) once we ascertain that p65 represses GADD153 expression under these conditions.

The following results with MCF10/p65NLS50 cells reveal a unique function for NF- κ B in breast cancer progression.

Epithelial to mesenchymal transition (EMT) is considered as an important step in cancer progression (10). It has been shown recently that epithelial cells that have undergone EMT serve as non-malignant stroma that supports growth of cancer cells (10). MCF10A cells overexpressing p65 showed hallmarks of epithelial-mesenchymal transition such as a fibroblastic phenotype, down-regulation of E-cadherin and Occludin, and up-regulation of Vimentin as measured by reverse transcriptase-polymerase chain reaction (RT-PCR) (Fig. 3A). We will perform additional in vitro studies with MCF10/p65NLS50 cells to determine its ability to promote growth of established cancer cells.

Snail family of transcription factors plays an important role in EMT (11). Several members of this family have been identified in mammalian cells (12). To determine whether NF- κ B mediated EMT correlates with increased Snail expression, we measured expression levels of Snail-1 and 2 by RT-PCR (Fig. 3B). While Snail-2 expression was not dependent on NF- κ B, Snail-1 expression was lower in p65 overexpressing cells, which is contrary to our expectation. We are currently investigating whether NF- κ B regulates Snail-3 expression.

During the course of this investigation, we made another observation that MMS but not other environmental toxicants induces Snail-1 expression in breast cancer cells as well as in MCF-10A cells (Fig. 3 and 4). This is an important observation considering the role of Snail in EMT and the effect of EMT on cancer progression. Although these studies are not part of the original proposal, they identify an important mechanism by which environmental toxicants can promote breast cancer.

Specific aim II:

Task 1: Determine the DNA binding pattern of C/EBP β and C/EBP β :GADD153 heterodimers in various cell types by EMSA.

Task 2: Determine the expression levels of C/EBP β and GADD153-responsive genes in various cell types by transient transfection, Northern and Western blots.

Task 3: Months: determine the differentiation pathway in various cell types in response to lactogenic stimulation. This will be achieved by measuring β -casein and WAP expression in response to prolactin treatment.

Results: Studies related to this aim has been initiated. Because of delay in hiring post-doctoral fellow and difficulties in establishing MCF10Ap65NLS50 cells, additional time may be required to complete these studies.

Specific aim III

Task 1: Establish cells overexpressing p53 dominant-negative mutants and characterize them for p53 activity. Stable cell lines overexpressing p53V144A will be established.

Task 2: Determine MMS sensitivity, NF- κ B activity and GADD153 expression in various cell types.

Task 3: Determine the MMS-induced transformation frequency of cells that express p53 dominant negative mutant.

Results: Most of the studies of this aim are planned for the third year of the grant.

However, we have already prepared plasmids to generate cells overexpressing dominant negative p53. We have also standardized siRNA technique in the lab to deplete p53 from MCF10A cells, which may be more efficient than dominant negative mutants in suppressing p53 activity. Furthermore, we have obtained a chemical inhibitor of p53 to reduce p53 activity in MCF-10A cells.

In the first annual report, we provided preliminary data on the mechanism of p65-mediated suppression of GADD153 expression. Results of subsequent studies, which further explain the mechanisms, are summarized below.

1. We previously showed that p65 represses GADD153 promoter activity using transient transfection assay with a CAT reporter gene under the control of GADD153 promoter extending to exon 1 (pG3/CAT) (Fig.5).
2. Deletion of the 91 bp Exon 1 region of GADD153 (pG3mut1/CAT) removes p65-mediated inhibition of CAT reporter activity. Cloning of Exon 1 upstream of the GADD153 promoter (p91/CAT) restored p65-mediated inhibition of CAT activity, but not an unrelated 91 bp sequence located within the GADD153 gene (pcontr91/CAT). Exon 1 contains a transferable p65 repressible element as an RSV promoter/CAT reporter with exon 1 of

- GADD153 but not unrelated 91 base long sequence was repressed by p65 (Fig. 6).
3. Determined that the p65-repressible element is located within a 30 bp fragment of the 91 bp exon 1 (+41 to +71 bp) region of the GADD153 gene (Fig. 7).
 4. A mutant of p65 with reduced transactivation potential, derived by site-directed mutagenesis, was less efficient in repressing GADD153 expression compared to wild type p65. These results suggest that a protein whose expression is induced by p65 is responsible for the repression of GADD153 expression (Fig. 8).
 5. The 30 bp region contains a putative binding site for the Gfi repressor family. Snail-1, a member of the Gfi, family is a transcriptional repressor. Electrophoretic mobility shift assay (EMSA) showed specific binding of Snail-1 to the 91 bp region (Fig. 9). In transient transfection assays, Snail-1 repressed GADD153 expression (Fig. 10).
 6. We did not observe direct binding of the p65 subunit to the 91 bp DNA sequence by EMSA. Furthermore, based on competition studies, transcription factors SP-1, AP-1, CREB, Oct-1 and E2F-1 do not appear to bind to the 91 bp sequence (Fig. 11).

KEY RESEARCH ACCOMPLISHMENTS:

- MCF-10A cells overexpressing p65 subunit of NF- κ B show an epithelial to mesenchymal transition phenotype.
- MCF-10A cells overexpressing p65 show reduced induction of GADD153 in response to MMS treatment.
- MMS induces the expression of Snail-1, which is linked to epithelial to mesenchymal transition.
- Snail-1 represses GADD153 expression.

REPORTABLE OUTCOMES:

Abstract: An NF- κ B repressible element in the 5' untranslated region of the proapoptotic GADD153/CHOP gene. Hui Lin Chua and Harikrishna Nakshatri. American Association for Cancer Research 94th Annual Meeting Washington, DC, June 10-14, 2003.

CONCLUSIONS: There has been a considerable progress in the last year. We have established MCF-10A cells overexpressing p65. Unexpectedly, overexpression of p65 alone was found to be sufficient for EMT phenotype of these cells. This is an important observation considering a well-established role for EMT in breast cancer progression. Most importantly, we have observed induction of Snail-1, a transcription repressor involved in EMT, by the environmental toxicant MMS. Although we have not been able to show definitive role for NF- κ B in suppressing environmental toxicant-induced GADD153 expression, preliminary results supports such a possibility. The effect of environmental toxicants on EMT through induction of Snail needs a through investigation in future as this may have an impact not only on cancer progression but also on the response of cancer cells to therapy.

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Fig. 1(A)

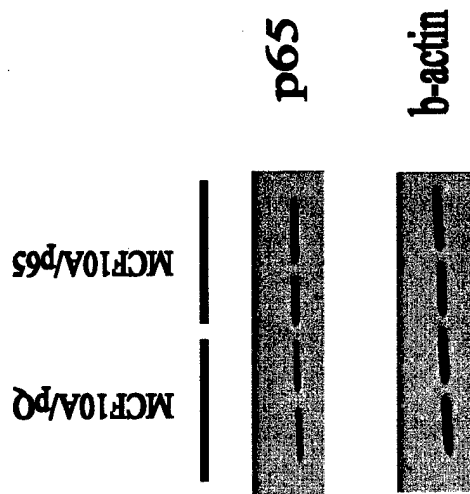


Fig. 1(A). MCF10/p65NLS50 cells express higher levels of p65 compared to MCF10A/pQ cells, as determined by Western blot analysis. Two mass cultures of each cell line were analyzed.

Fig. 1(B)

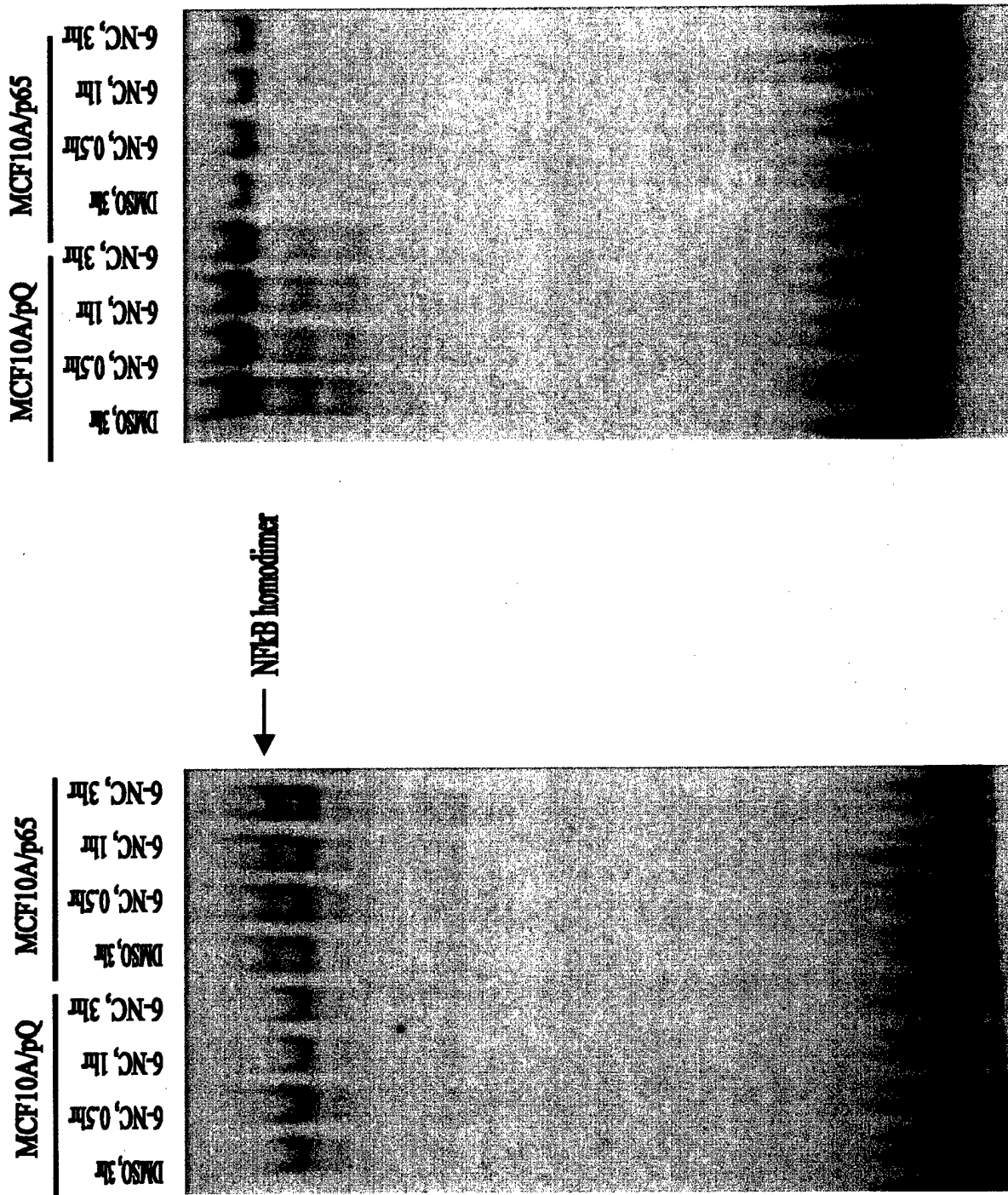


Fig. 1(B). Electrophoretic mobility shift assay (EMSA) analysis showed increased NFkB binding independent of 6-nitrochrysene (6-NC), in MCF10Ap65/NLS50 compared to MCF10A/pQ vector control cells, when nuclear extracts from indicated cell lines were incubated with a labeled NFkB probe. As a control, EMSA was also carried out by incubating samples with a labeled SP-1 probe.

Fig. 2(A)

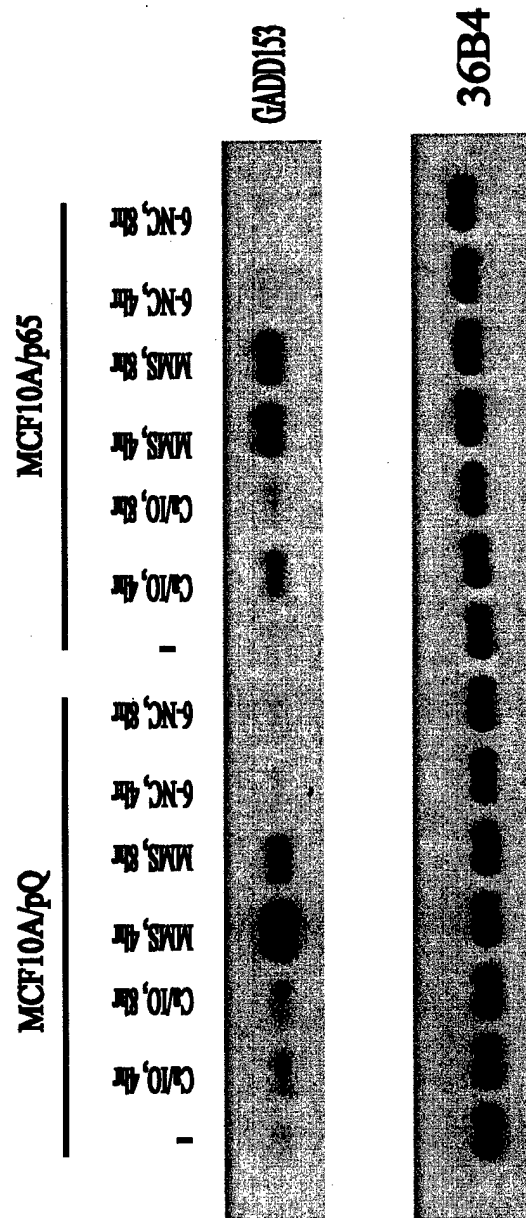


Fig. 2(A). GADD153 expression in MCF10A/pQ and MCF10A/p65 cells as measured by Northern analysis. MCF10A/pQ cells show induced GADD153 expression following treatment with MMS for 4 hrs. MCF10/p65NLS50 cells, however, showed a lower level of GADD153 induction compared to MCF10A/pQ cells. GADD153 expression does not appear to be appreciably induced by calcium-ionophore (Ca/IO) treatment in both cell lines. 6-nitrochrysine (6-NC), another environmental toxicant, had no effect on GADD153 expression. The same blot was reprinted with 36B4. A ribosomal protein gene, to ensure quality of RNA and equal loading.

Fig. 2(B)

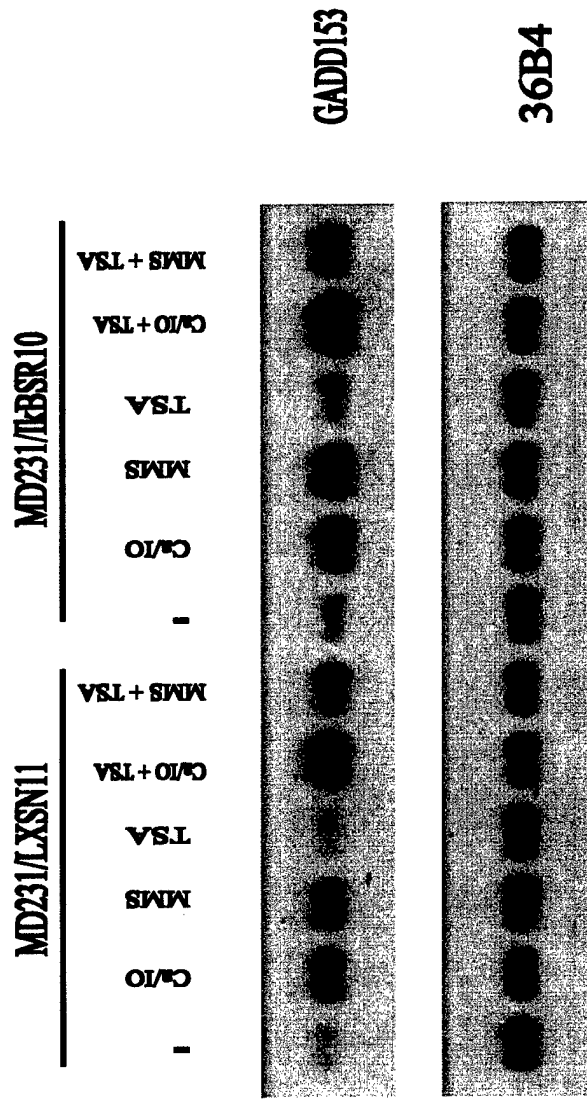
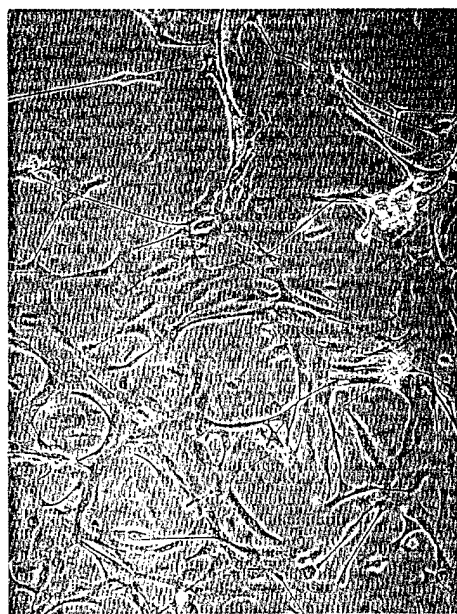


Fig. 2(B). MD231/lkBSR10 cells, which had lower NFkB than MD231/LXSN11 cells, show an increased level of GADD153 expression induced by calcium ionophore and MMS compared to MD231/LXSN11 cells. Addition of the histone deacetylase inhibitor, TSA, leads to a further increase in GADD153 expression induced by calcium ionophore. A similar induction of GADD153 expression was not observed following MMS and TSA treatment.

Fig. 3(A)



MCF10A/pQ



MCF10A/p65NLS50

Fig. 3(A). MCF10A/p65NLS50 cells show evidence of epithelial-mesenchymal transition as these cells exhibit a fibroblastic phenotype compared to MCF10A/pQ cells, which retain an epithelial phenotype.

Fig. 3 (B)

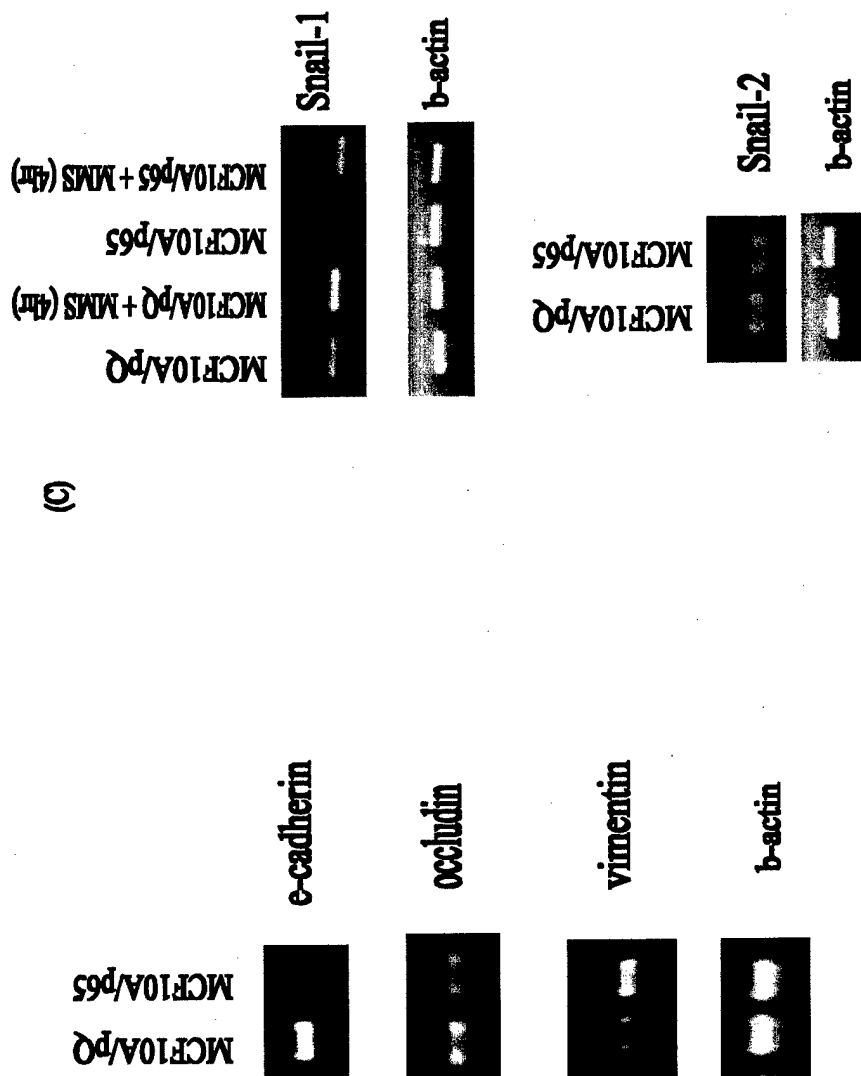


Fig. 3(B). The epithelial-mesenchymal transition exhibited by MCF10A/p65NLS50 cells is further supported by the down-regulation of epithelial markers including e-cadherin and occludin; and the up-regulation of vimentin compared to MCF10A/pQ cells, as determined by RT-PCR analysis. (C) Initial RT-PCR analysis showed that expression of Snail-1 was down-regulated in MCF10A/p65NLS50 compared to MCF10A/pQ cells. In addition, Snail-1 expression was up-regulated following MMS (500 uM) treatment. Snail-2 levels were similar in both cell types and Snail-3 was not detectable by RT-PCR.

Fig. 4

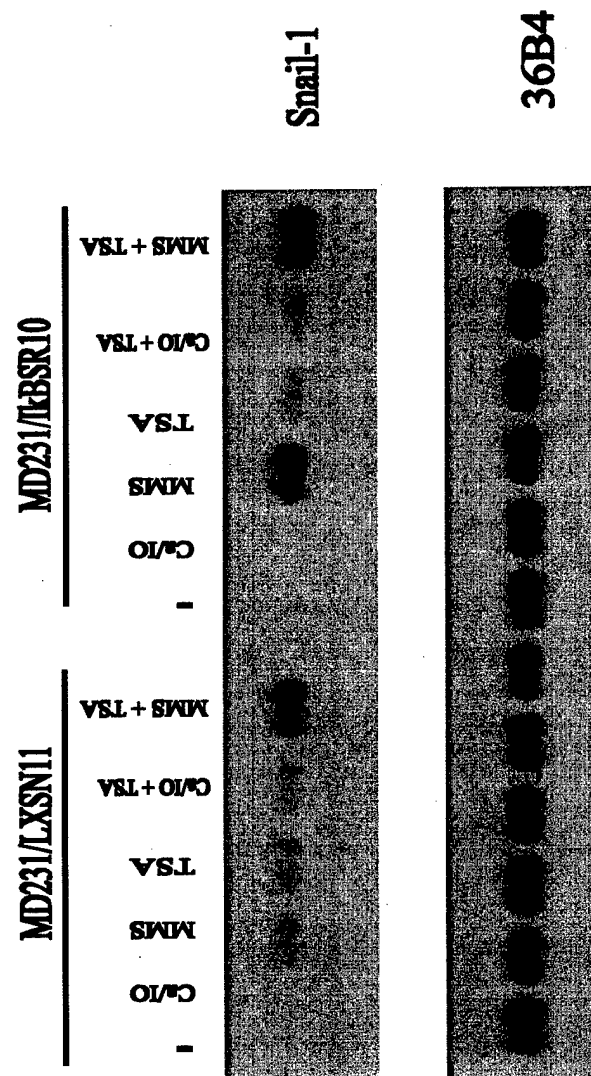


Fig. 4. Snail-1 expression was induced in MD231/kBSR10 cells following MMS treatment, as determined by Northern blot analysis.

Fig. 5

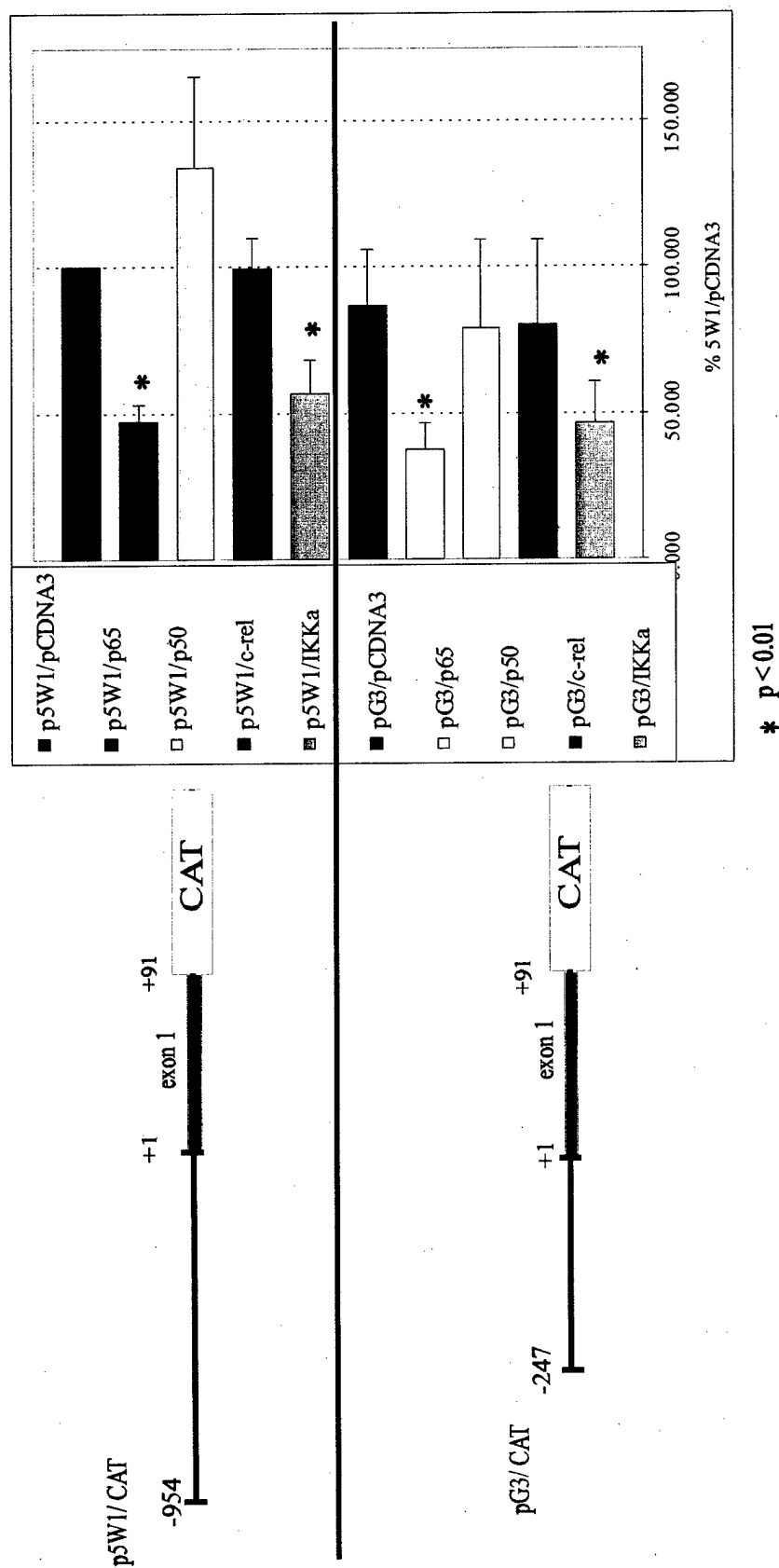


Fig. 5. p65 (but not p50) and IKK-alpha inhibit GADD153 promoter activity. COS-1 cells were transiently transfected with GADD153 promoter/CAT reporter constructs; and indicated concentrations of pCDNA3 (empty vector), p65 or p50 expression vectors. The beta-galactosidase expression vector pCH110 was co-transfected with to monitor transfection efficiency. CAT activity was measured 48 hrs after transfection based on an equal number of beta-galactosidase units.

Fig. 6

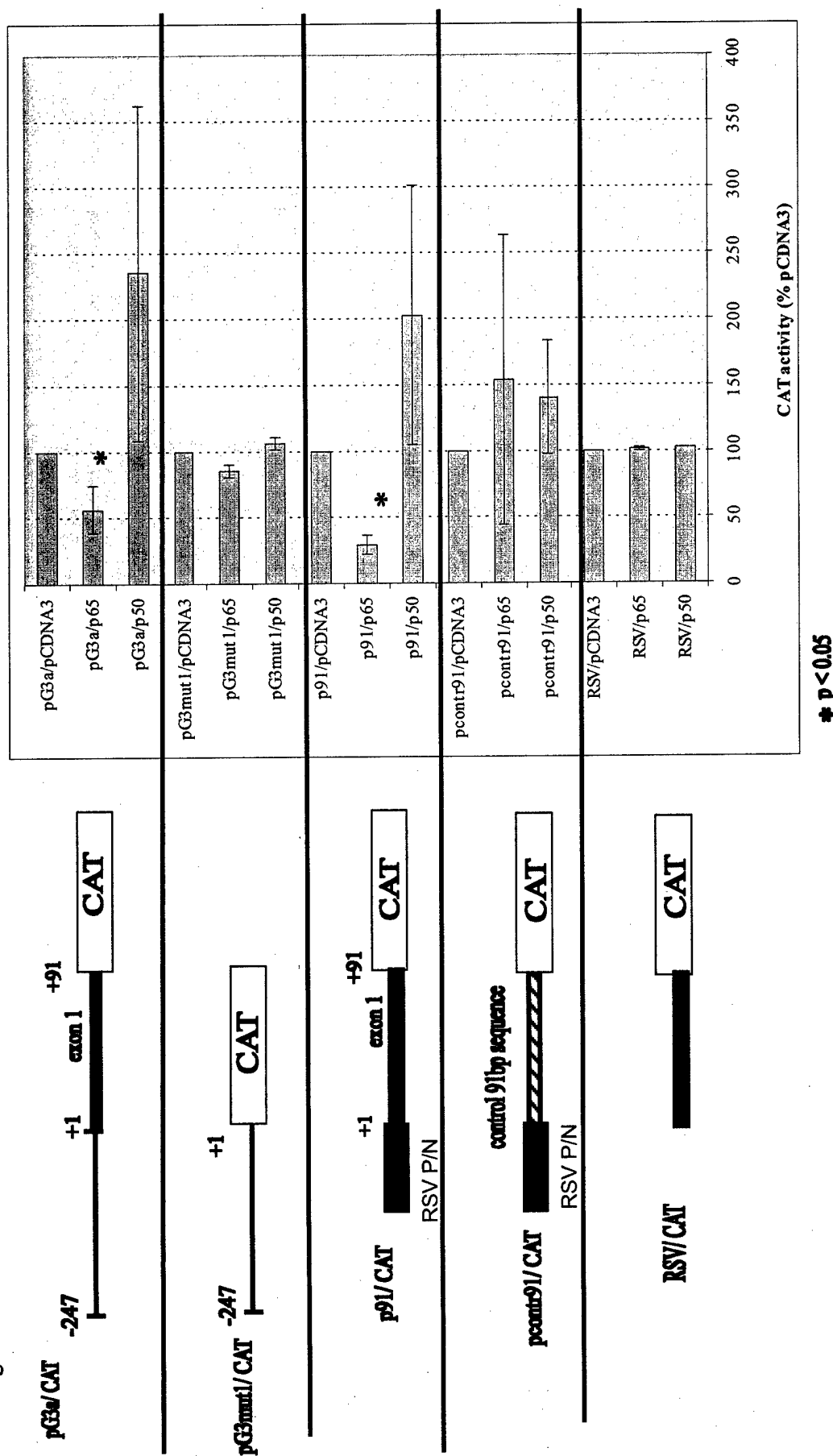


Fig. 6. Exon 1 of the GADD153 gene contains the p65-repressible element. Deletion of the 91 bp sequence (pG3mut1/CAT) removes p65-mediated inhibition of CAT reporter activity. Cloning of Exon 1 upstream of the CAT gene (p91/CAT) led to p65-mediated inhibition of CAT activity, but not an unrelated 91 bp sequence located within the GADD153 gene (pcontrol91/CAT). The 91 bp sequences corresponding to Exon 1 or unrelated DNA were cloned into the RSV/CAT expression vector.

Fig. 7

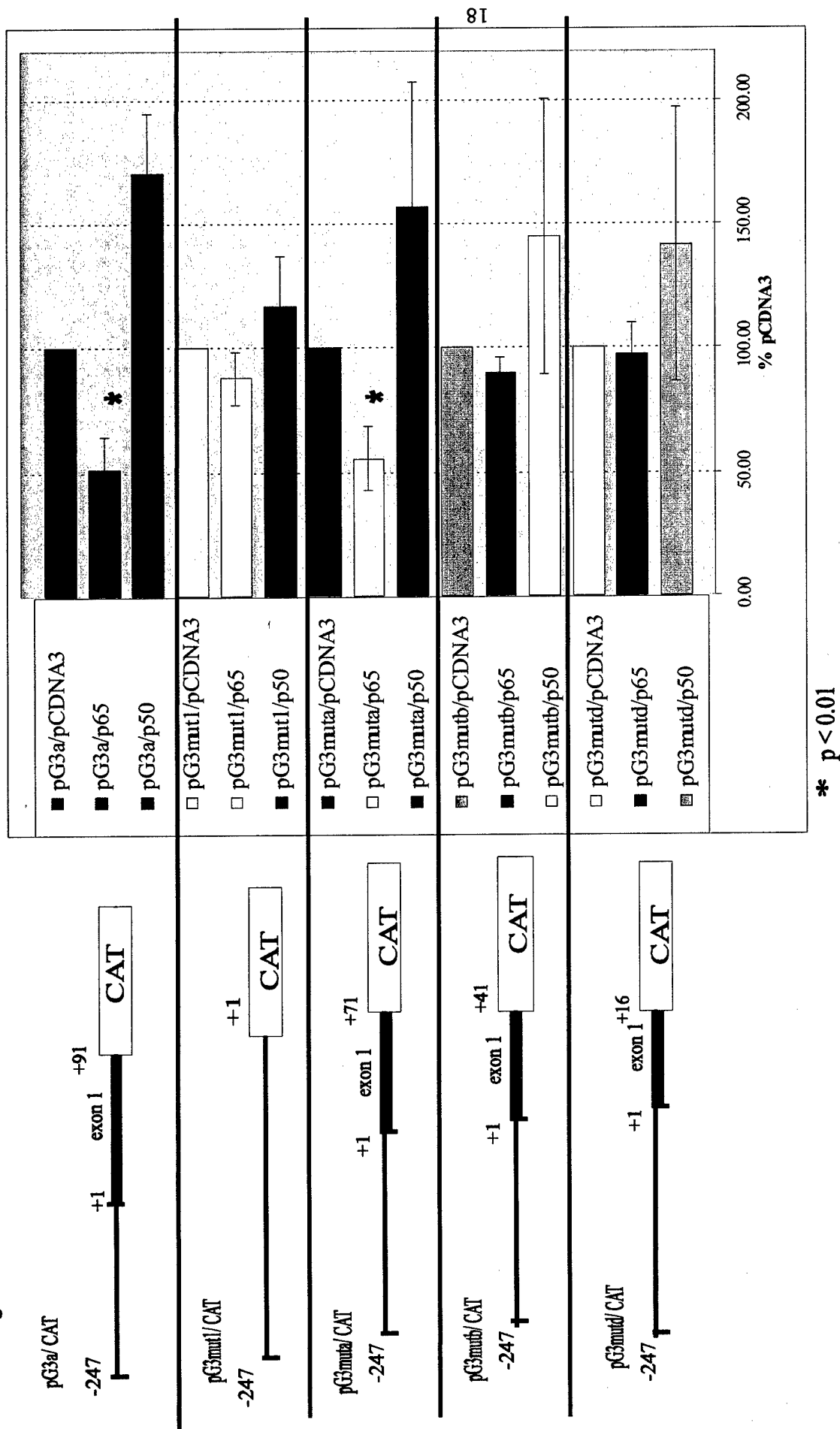


Fig. 7. The p65-repressible element is located in a 30 bp fragment (+41 to +71) within exon 1 of the GADD13 gene.

Fig. 8

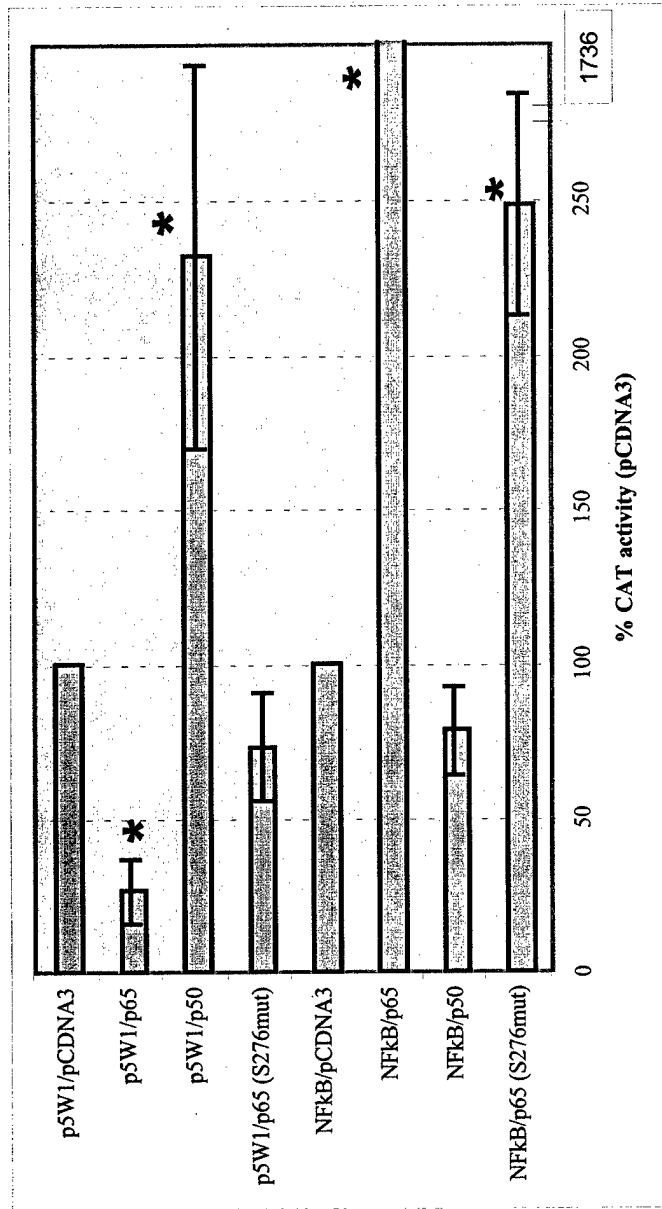
* $p < 0.01$

Fig. 8. A mutant form of p65 (S276mut) containing a serine to alanine mutation at position 276, located in the Rel homology domain, did not show significant repression of GADD153 promoter activity compared to wild-type p65. In addition, S276mut was less efficient in increasing transcription from a NFkB-responsive element-dependent reporter.

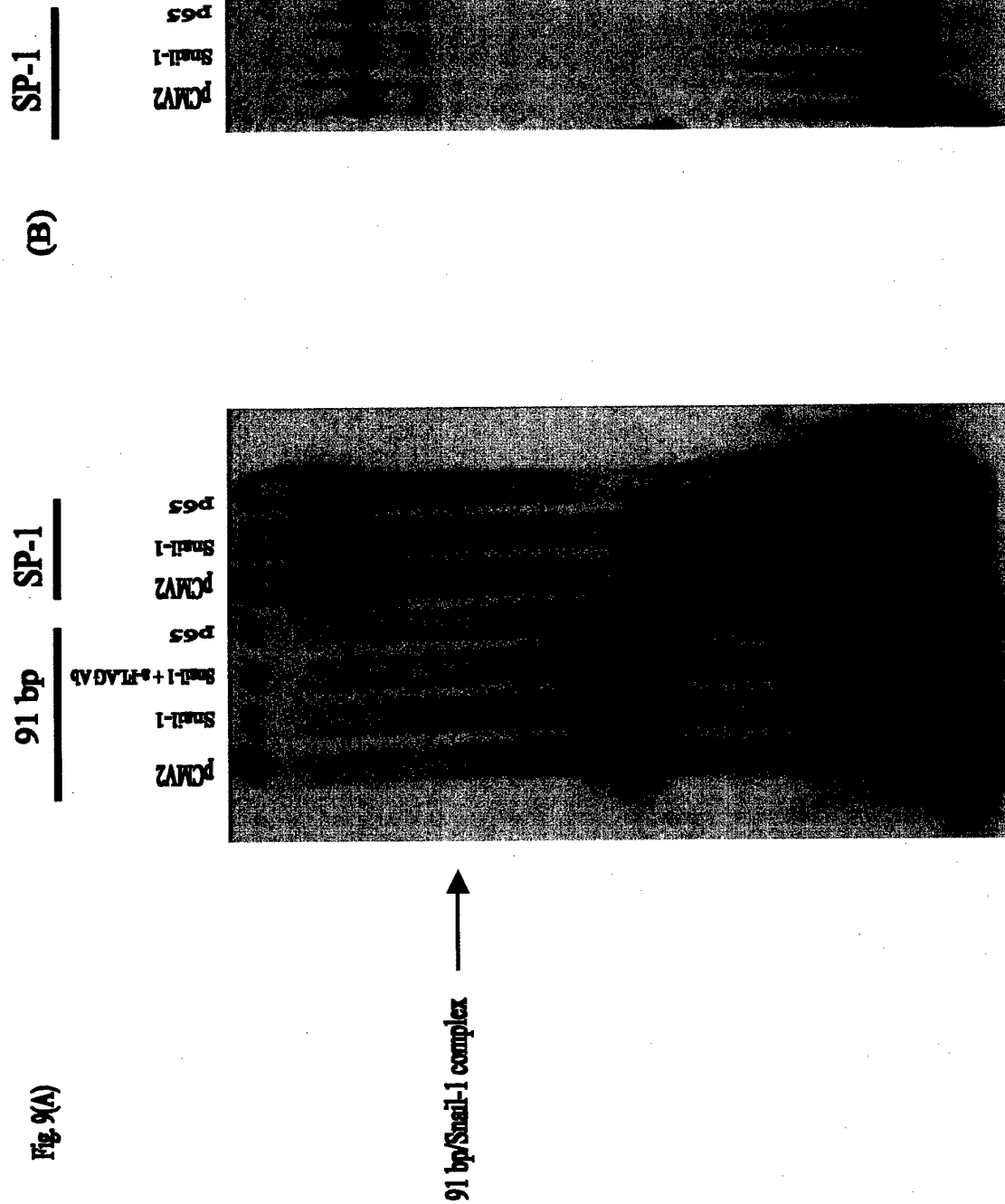


Fig. 9(A). Snail, a transcription repressor binds to NFkB repressible element. Extracts from COS-1 cells transiently transfected with pCMV2, pCMV2/Snail-1 or p65 expression vectors were analyzed by EMSA using the 91 bp exon 1 element or SP-1 as a probe. A 91 bp/Snail-1 complex was detected in cells transfected with the Snail-1 expression vector, but was no longer observed when anti-FLAG antibody was included in the reaction. (B) A shorter exposure of cell extracts incubated with the SP-1 probe was obtained.

Fig 10(A)

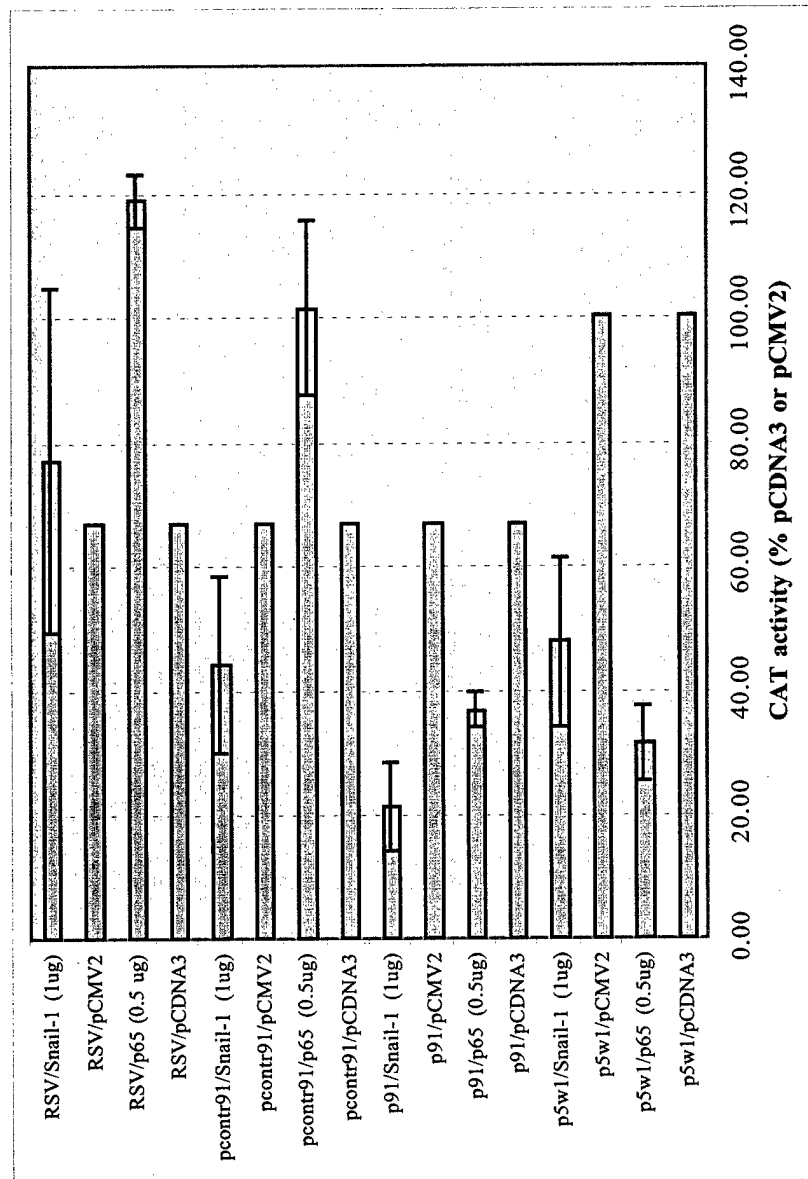


Fig. 10(A). Transient transfection of COS-1 cells with the Snail-1 expression vector led to inhibition of the full-length GADD153 promoter (p5W1)/CAT construct, as well as the CAT construct containing the 91 bp sequence (p91), but not an unrelated 91 bp (pcontr91) sequence.

Fig. 10 (B)

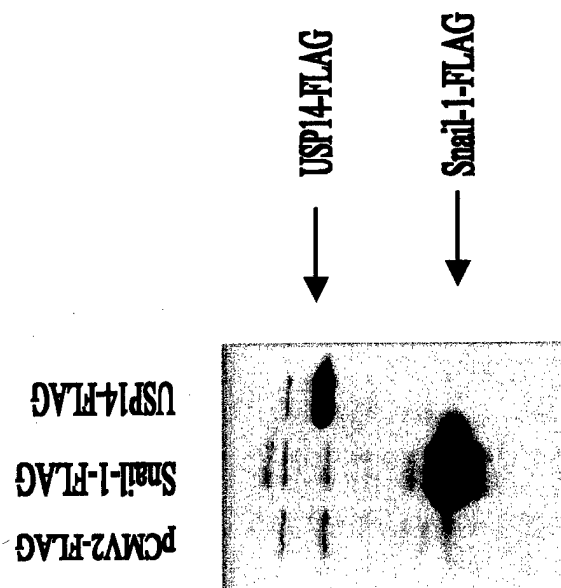


Fig. 10 B). Western blot analysis of COS-1 cell extracts showed overexpression of Snail-FLAG protein in cells transiently transfected with the Snail-FLAG expression vector. Transient transfection of COS-1 cells with the USP14-FLAG expression vector was also carried out.

Fig. 11(A)

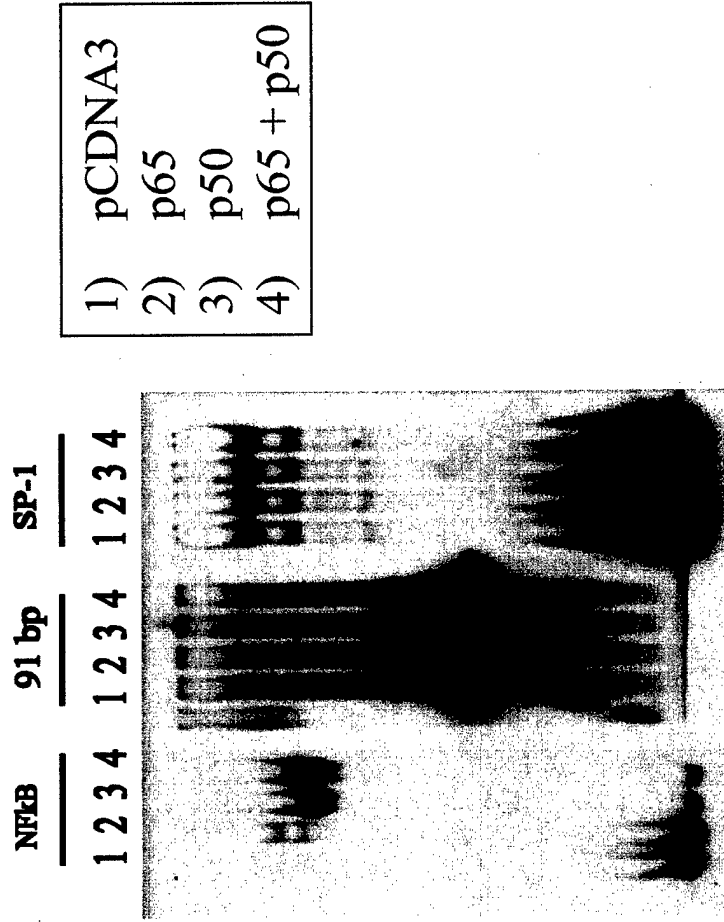


Fig. 11(A). p65 does not bind to the 91 bp sequence. EMSA was carried out using cell extracts obtained from COS-1 cells transiently transfected with pCDNA3, p65 and/or p50 expression vectors.

cold competing nucleotide
 E2F-1
 Oct-1
 AP-1
 CREB
 SP-1
 NFkB
 91 bp

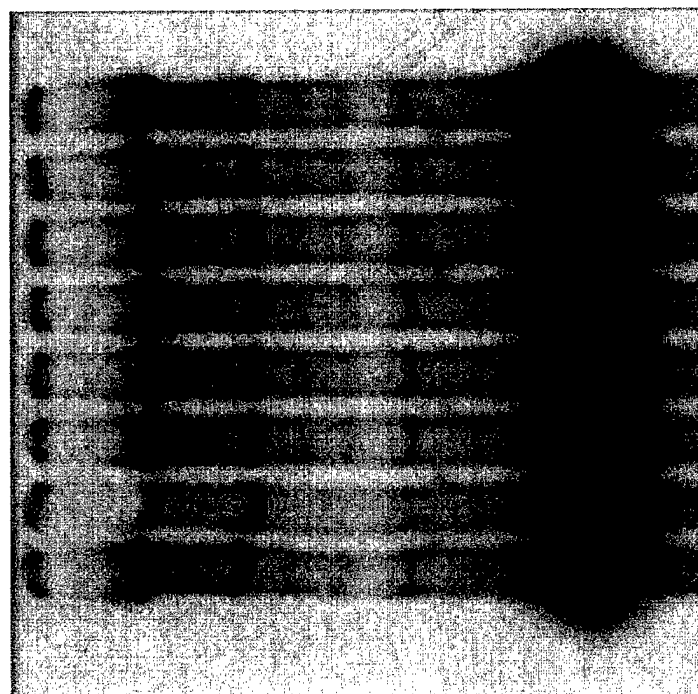


Fig. 11(B)

Fig. 11(B). The factors NFkB, SP-1, CREB, AP-1, Oct-1 and E2F-1 are not likely to bind to the 91 bp sequence. Whole cell extracts from COS-1 cells were incubated with radiolabeled 91 bp probe as well as unlabeled competing oligonucleotide.

rDNA transcription during differentiation. These data indicate that members of the MAX-network of transcription factors can regulate rDNA transcription and cell growth providing a mechanism to link the regulation of cell growth to the cell division cycle during differentiation.

#2376 Modulation of the folate receptor type β gene by coordinate actions of retinoic acid receptors at activator Sp1/ets and repressor Ap-1 sites. Hong Hao, Huiling Qi, and Manohar Ratnam. *Medical College of Ohio, Toledo, OH.*

Folate receptor (FR) type β is a promising target for therapeutic intervention in acute myelogenous leukemia (AML) owing particularly to its specific upregulation in AML cells by all-trans retinoic acid (ATRA) (Wang et al, 2000, *Blood*, 96:3529-3536; Pan et al, 2002, *Blood*, 100:594-602). Here we identify functional elements in the FR- β gene and examine the molecular mechanism of transcriptional induction of FR- β by ATRA. The basal promoter activity of FR- β resulted from synergistic interaction between Sp1 and ets binding (EBS) elements and repression by upstream AP-1-like elements, whose action required EBS. A minimal promoter containing the Sp1 and ets elements was ATRA-responsive. The repressor elements bound Fos family proteins; association of the proteins with the repressor elements correlated negatively with FR- β expression in peripheral blood neutrophils and monocytes and also in KG-1 (AML) cells grown in the absence or in the presence of ATRA. Furthermore, downregulation of FR- β in KG-1 cells treated with O-tetradecanoylphorbol 13-acetate (TPA) was accompanied by increased AP-1 binding to the repressor elements. From chromatin immunoprecipitation (ChIP) assays, the nuclear receptor RAR α associated with the Sp1 region and RARs β and γ associated with the AP-1 and Sp1 regions; treatment of KG-1 cells with ATRA did not alter Sp1 binding but increased the association of RAR α and decreased the association of RARs β and γ . ATRA also decreased RAR expression levels. The results suggest that the FR- β gene is a target for multiple coordinate actions of nuclear receptors for ATRA directly and indirectly acting on a transcriptional complex containing activating Sp1/ets and inhibitory AP-1 proteins. The multiple mechanisms favor the prediction that ATRA will induce FR- β expression in a broad spectrum of AML cells. Further, optimal FR- β induction may be expected when all three RAR subtypes bind agonist.

#2377 An NF- κ B-repressible element in the 5'-untranslated region of the pro-apoptotic GADD153/CHOP gene. Hui Lin Chua and Hari Krishna Nakshatri. *Indiana University School of Medicine, Indianapolis, IN.*

Cells exposed to stresses such as nutrient deprivation, hypoxia, ultraviolet light and agents that damage DNA or disrupt endoplasmic reticulum function show induced expression of GADD153/CHOP. GADD153 is a member of the C/EBP family of transcription factors and promotes apoptosis following cellular stress. Interestingly, cellular stress also induces NF- κ B, which promotes cell survival by inducing transcription of a number of anti-apoptotic proteins, such as Bcl-2, Bcl-XL, x-IAP and c-IAP-2. GADD153 mRNA expression was previously shown in this laboratory to be inhibited by the p65 transactivating subunit of NF- κ B. Therefore, cells expressing constitutively active NF- κ B, as is the case in 30% of breast cancers, may be predisposed to transformation or malignancy, due to p65-mediated inhibition of GADD153 expression following cellular stress. By transient transfection assays, we identified an NF- κ B-repressible element (NRE) within a 91-base pair portion of the 5'-untranslated region of the GADD153 gene. In addition, transient transfection of p65 with various co-activators or co-repressors failed to reverse the inhibitory effect. Insertion of GADD153 NRE, but not an unrelated 91-base pair sequence, to the 5'-untranslated sequence of the Rous Sarcoma Virus enhancer/promoter-CAT reporter (RSV/CAT) led to p65-dependent repression of RSV/CAT expression. Interestingly, the same region of GADD153 has been previously shown to be required for UVC-mediated repression of stress-inducible GADD153 expression. We did not observe direct binding of the p65 subunit or any p65-inducible proteins to the 91-base pair DNA sequence by electrophoretic mobility shift assay. Based on a previous observation of MyoD mRNA destabilization by p65, we speculate that a p65-inducible protein destabilizes GADD153 mRNA by binding directly to the 5'-untranslated region of GADD153 mRNA. Understanding the mechanisms by which NF- κ B mediates inhibition of GADD153 expression will facilitate the design of strategies to promote apoptosis of damaged cells thereby preventing initiation of transformation.

#2378 AP-1 blockade inhibits breast cancer cell growth by preventing the recruitment of coactivators. Lu Chunhua, Qiang Shen, Heetae Kim, David De-nardo, Kendall Wu, and Powel Brown. *Baylor College of Medicine, Houston, TX.*

The AP-1 transcription factor is a central component of signal transduction pathways in many cells. We have previously demonstrated that blocking AP-1 by over-expressing a dominant negative form of cJun (cJun-DN, TAM67) inhibits breast cancer cell growth. We hypothesize that TAM67 blocks AP-1 causing breast cancer cell growth by preventing the coactivators that bind AP-1 transcription

factors dimerization complex. In the present study, we used two clones of MCF7 breast cancer cells that express either flag-tagged TAM67 or cJun under the control of an inducible promoter. First, we compared the ability of cJun and TAM67 to bind coactivators, such as JAB1, AIB1, CBP and P300, in MCF7 cells by using immunoprecipitation-Western blotting techniques. We observed that the coactivators (JAB1, AIB1, CBP, P300) all co-precipitate with c-Jun in MCF7 cells. In cells expressing TAM67, AIB1, CBP, P300 failed to co-precipitate with TAM67, however JAB1 did co-precipitate with TAM67. We are now performing ChIP assays using primers specific for the collagenase and cyclinD1 promoters. To determine whether TAM67 binds to these promoters and alters recruitment of coactivators. Our data suggests that TAM67 inhibits breast cancer cell growth by preventing the recruitment of coactivators, leading to suppression of AP-1 dependent gene expression. The present results also provide the foundation to develop selective AP-1 inhibitors that may be useful future drugs for the treatment or prevention of breast cancer.

#2379 Thymosin b-10 binds E-tropomodulin and induces an apoptosis by blocking the formation of actin-binding complex. Seung Bae Rho, Taehoon Chun(*), Seung-hoon Lee, and Je-Ho Lee. *Molecular Therapy Research Center, Samsung Medical Center, Seoul, South Korea and Department of Microbiology and Immunology, College of Medicine, Hanyang University, Seoul, South Korea.*

Thymosin b-10 (TB10) is a small G-actin-binding protein that induces depolymerization of intracellular F-actin pools by sequestering actin monomers. Previously, we demonstrated that overexpression of TB10 leads to an increased frequency of apoptosis. As an initial step to define molecular mechanism of TB10 dependent apoptotic process, we searched a human ovary cDNA library for a novel TB10 binding protein using a yeast two-hybrid system. As bait, we used the full length of human TB10 cDNA. The selected protein represented the C-terminal 291 amino acids of human E-tropomodulin (E-Tmod), another component of the actin-binding complex. Specific interaction of the E-Tmod with TB10 was confirmed by in vitro pull-down assays. The smallest structural unit of the two interacting protein components was determined quantitatively via in vivo analysis. Our results showed that the full length of TB10 is required to bind with E-Tmod. In addition, the TB10 binding site on E-Tmod at amino acid position 162-234 partially overlaps with the actin binding site at amino acid position 190-359. Furthermore, introduction E-Tmod cDNA into a tumor cell line reversed TB10 dependent apoptosis in a dose-dependent manner. These results strongly support that TB10 regulates apoptotic homeostasis by not only just binding to actin but also competing or blocking the protein complex formation of E-Tmod with actin.

#2380 Inhibition of Multiple Myeloma Cell Proliferation By TRAF6 Silencing mRNA. Haiming Chen, Hank Yang, Kim Burkhardt, Guangxu Li, Robert Lieberman, Daocheng Zhu, Xiamin Yan, Robert Vescio, and James R. Berenson. *Cedars-Sinai Medical Center, Los Angeles, CA.*

We previously have shown that increase in nuclear factor (NF)- κ B activity is associated with the enhanced tumor cell survival in MM cells. Increase in NF- κ B activity has also shown to induce tumor cell proliferation and metastasis. On the other hand, tumor necrosis factor receptor-associated factor (TRAF) 6 is essential for the activation of NF- κ B signaling in plasma cells and the Jun NH2-terminal kinase (JNK) pathway, which controls osteoclast proliferation. The pivotal role of TRAF6 in signal transduction provides an ideal target of inhibition in order to enhance the killing of MM cells and inhibit bone turnover mediated by osteoclast activity. RNA interference (RNAi) has emerged as a powerful tool that is used to block the expression of a specifically targeted gene. In this study, we used RNAi to specifically block TRAF6 mRNA expression and thereby inhibit the activation of NF- κ B. We created two human TRAF6 ds-RNAs by *in vitro* construction that target either a Zn-finger or a TRAF-C domain of TRAF6 protein. We also established a stable 8226 MM cell line through transfection that expresses EGFP. The EGFP-transduced MM cells can be easily tracked down and enriched by FACS sorting for EGFP-positive cells. We demonstrated that there is no difference in apoptosis and cell proliferation between EGFP-positive and -negative MM cells. RT-PCR and Western blotting showed that TRAF6 gene expression was greatly reduced by RNAi of TRAF-C domain. However, introduction of RNAi Zn-finger sequence into 8226 cells failed to inhibit TRAF6 production. NF- κ B mRNA level was also reduced by RNAi of TRAF-C domain, but not by RNAi of Zn-finger domain. Cell proliferation assays showed that RNAi of the TRAF6 C-domain inhibited MM cell proliferation in a dose-dependent manner when EGFP-labeled MM cells were co-cultured with human bone marrow stromal cells. The RNAi of the TRAF6 Zn-finger did not produce detectable inhibition of MM cell proliferation. This inhibition in cell proliferation correlated with an increase in apoptosis. RNAi of TRAF6 C-domain slightly increased the MM cell apoptosis. In contrast